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(54) Immunogenic preparations

(57) Antigenic glycolipoproteins isolable from the egg homogenate of fully fed female tick *Rhipicephalus appendiculatus* Neumann are useful in immunizing livestock and other hosts for the control of the tick in the field.

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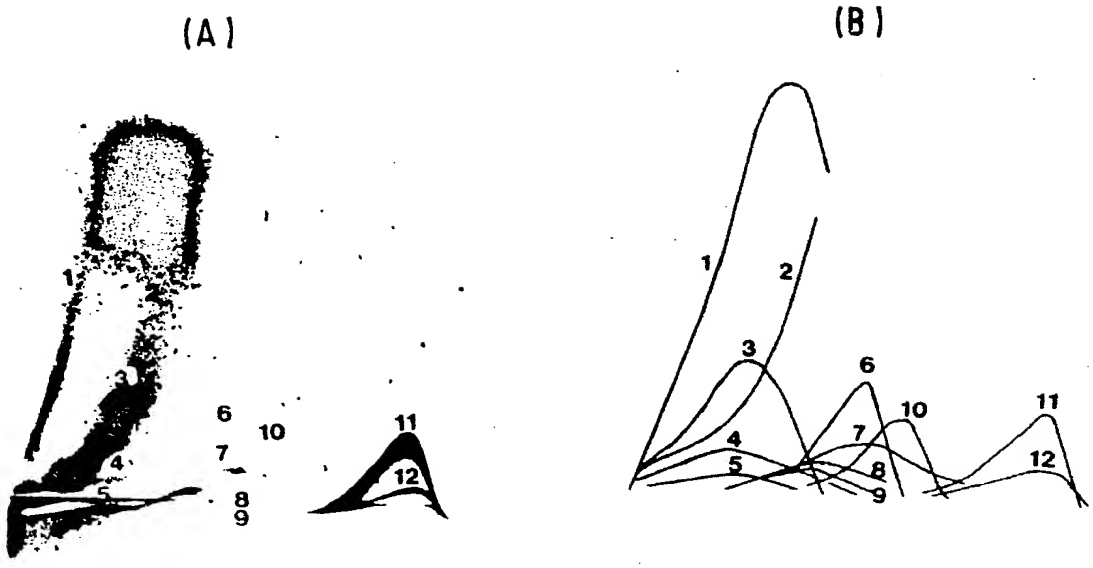

Fig.i.

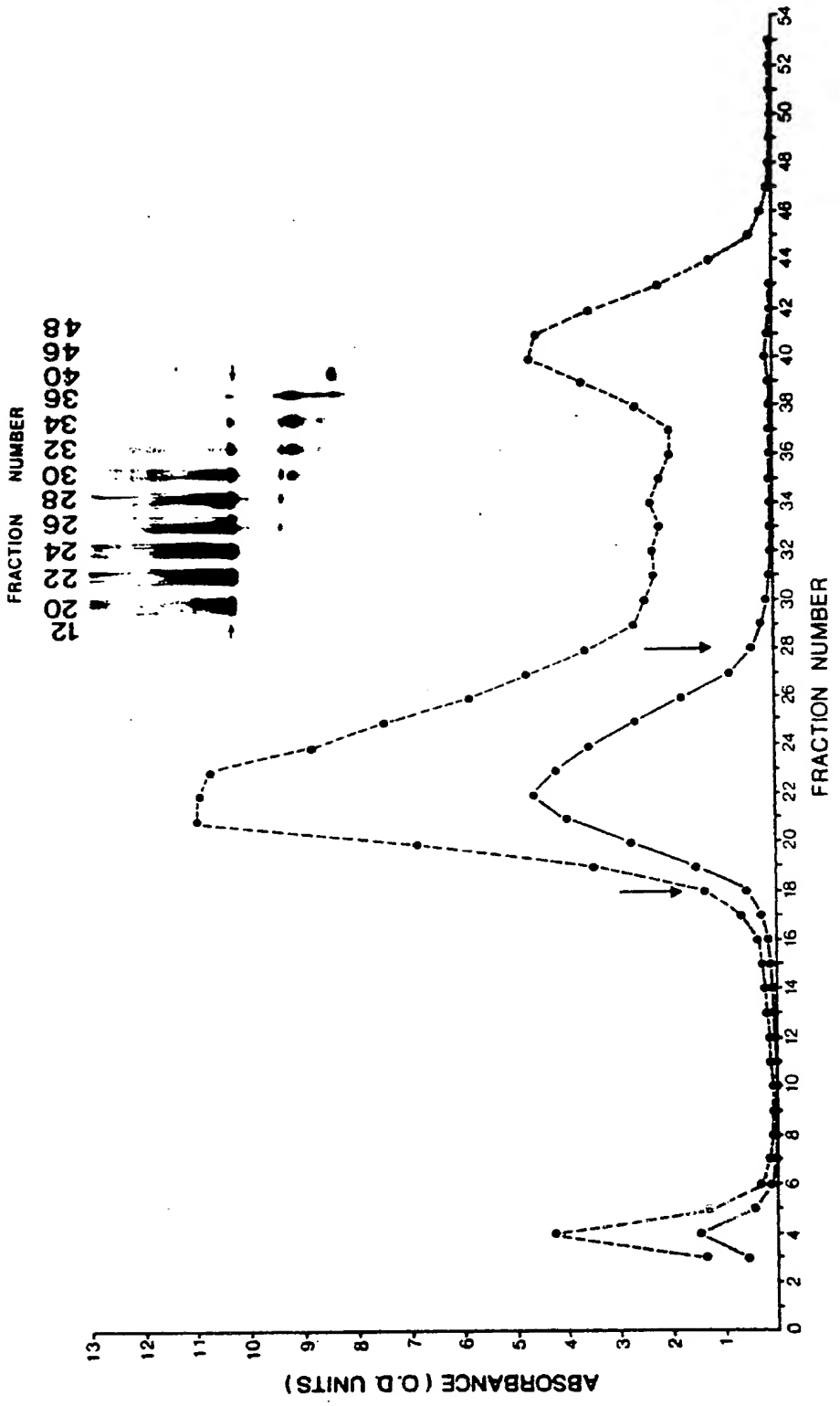

Fig.ii.

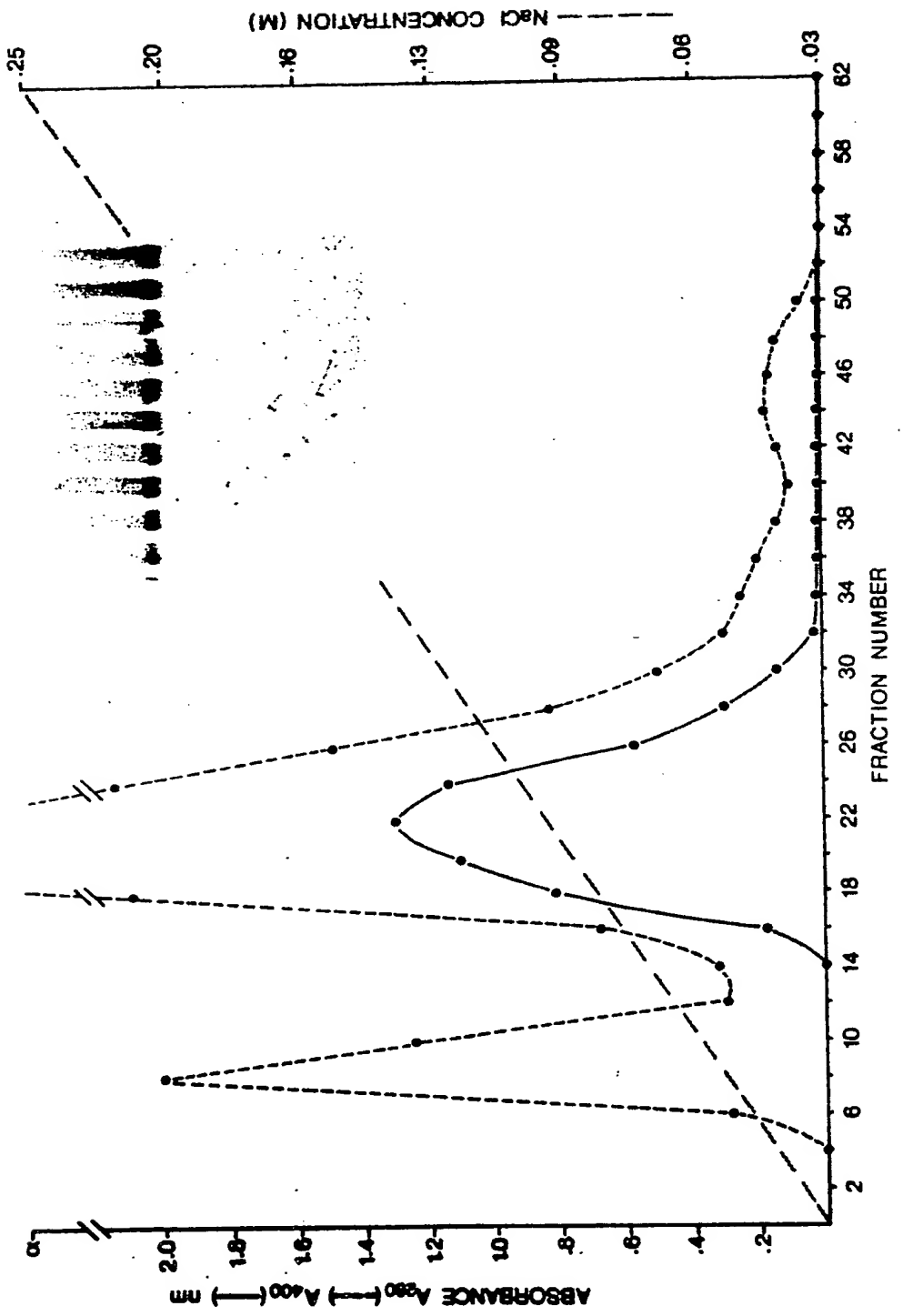

Fig. iii.

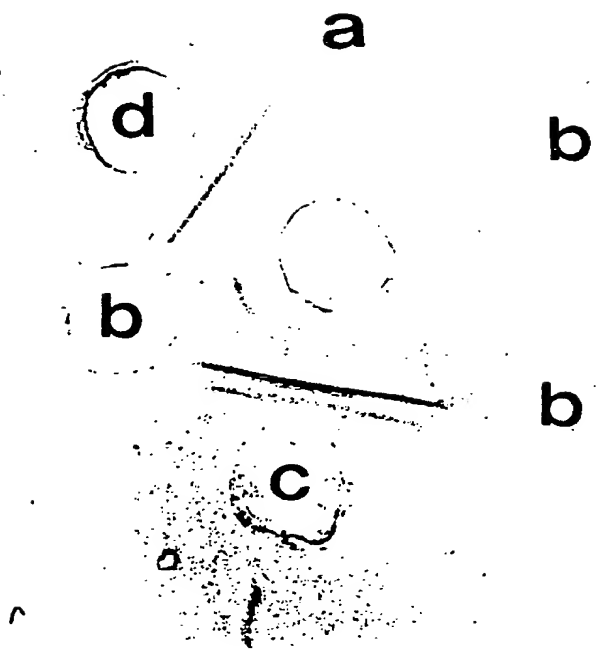

Fig. iv.

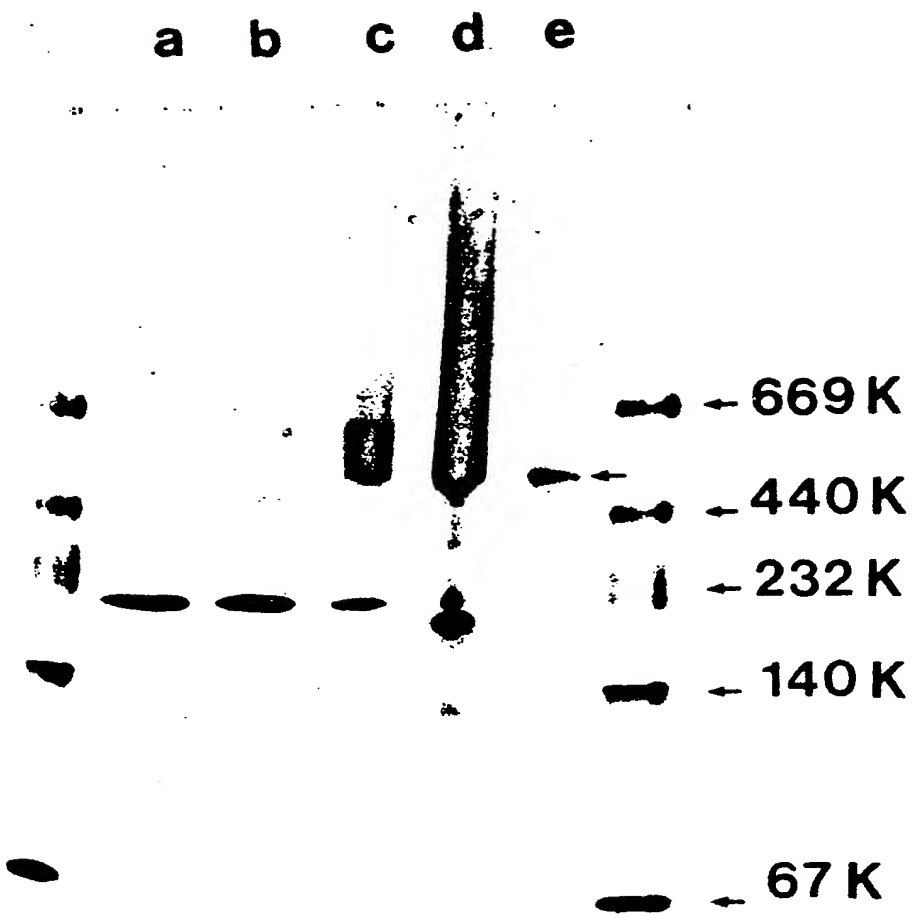

Fig.v.

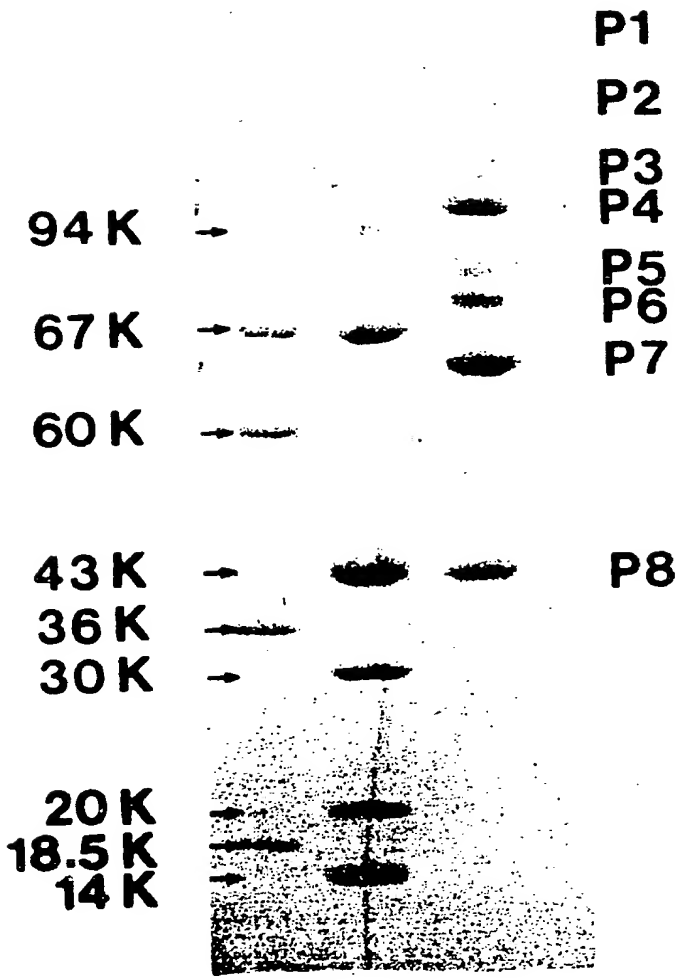

Fig. vi.

SPECIFICATION

Novel Immunogenic preparations

1. The present invention relates to novel antigen macrocompounds which can be used to immunise cattle against infestation by the ixodid tick, *Rhipicephalus appendiculatus* Neumann, the vector of the African cattle disease East Coast Fever. More particularly, the invention is concerned with an immunogen derived from eggs of the tick, *R. appendiculatus,* and with immunogenic compositions prepared therefrom, which, when inoculated into mammals, stimulate the production of antibodies which not only reduce feeding by the ticks but also interfere with normal growth, development and reproduction of the engorged ticks.

2. It is known that multiple tick infestation of cattle induce some resistance in these animals against further infestation, and that this resistance is associated with humoral immune components. It is further known that similar resistance can also be induced by immunizing cattle and other mammals with crude homogenates derived from different organs of partially fed or fully fed ticks. However, immunization methods based on crude antigenic preparations tried thus far have failed to outperform the limited resistance attainable natural as a result of repeated tick infestation.

3. It has been found that antisera obtained from animal such as rabbits immunized with crude homogenates of fully fed *R. appendiculatus,* conferred protection to immunized animals against feeding ticks. Cross immunoelectrophoresis showed the presence of at least 12 precipitin bands corresponding to different antigenic macrocompounds in the antisera.

4. The present invention is based on the isolation and identification of a female-specific immunogen present in homogenates of fed females of *R. appendiculatus* and is incorporated in the eggs, and on the finding that this immunogen, alone or in combination, provides improved protection against tick infestation.

5. According to the invention, there is provided a hemeglycolipoprotein with a native molecular weight of about 485 thousand Dalton units present as the major protein component in the eggs of *R. appendiculatus.* SDS—polyacrylamide gel electrophoresis showed that the protein is made up of 8 subunits with molecular weights of 160, 137, 110, 100, 85, 79, 66 and 43 thousand Dalton units, respectively.

It is to be understood that the primary immune-inducing entity may be either the native protein itself or one or more of the subunits derived from it. Accordingly, the term "antigen", "antigenic protein" or "immunogen" used in the present context are meant to include not only the native hemeglycolipoprotein with a molecular weight of about 485,000 Dalton units, but also any combination of its subunits.

6. The preferred but not exclusive source of the said antigen is the fully developed eggs of the ticks. It is to be understood, however, that tissues or individual organs derived from partially fed or fully fed ticks could be used as sources of the said antigen. Separation and purification of the said antigen can be achieved by gel permeation and ion-exchange chromatography. It may also be achieved by a variety of other techniques, such as affinity chromatography, preparative electrophoretic and chromatofocussing techniques, or a combination of these methods. The preferred method for medium—and large-scale separation and purification of the antigen is based on the use of biospecific adsorbents, such as commercially available protein A—sepharose CL–4B linked to monoclonal antibodies raised against the said antigen.

Large scale synthesis of the antigen may also be achieved by use of the available recombinant DNA techniques, whereby the cloned gene(s) containing coding sequence(s) for the antigen may be incorporated into the genome of a suitable vector for continued *in vitro* expression of the antigen.

7. As an additional feature of the invention, there is provided a method for artificial induction of resistance in mammals to the tick *Rhipicephalus appendiculatus* by inoculating the host animals with a formulation made up of the said antigen with a suitable adjuvant. A particularly potent immunogenic preparation involves complexing the said antigen, alone or in a selected combination, with antibodies derived from immunized animals and formulating the resulting antigen-antibody complex(es) with a suitable adjuvant.

8. The invention is illustrated, but not limited, by the examples below describing the preparation of antiserum, purification of the said antigen and immunisation studies.

The examples refer to Figs. i to vi of the accompanying drawings.

Figure i: (A) shows results of crossed immune electrophoresis of an extract of fed female R. *appendiculatus* with serum from a rabbit immunized with homogenate of fed female ticks.

Figure i: (B) shows tracing of the stained pattern in (A). the upper gel contained antiserum against fed tick antigens; 1.25 mg equivalent of fed tick protein was electrophorosed in the lower, first dimension gel. In this and Fig. 2 the cathode was to the left for the first electrophoresis and at the bottom for the second electrophoresis.

Figure ii relates to the elution profile of soluble protein extract of *R. appendiculatus* eggs from

sepharose GL–4B column. The egg proteins were eluted with TNE buffer (0.15M Trisc–HCl, 0.1M NaCl, 0.1M EDTA and 0.02% NaN_3, pH 8.0) and 3.1 ml fractions collected. The absorbance of each protein fraction was monitored at 280 nm (o . . . o) and 400nm (o—o) on a Beckman SP500 spectrophotometer. The inset is a photograph of a native polyacrylamide gel electropherograms, stained for proteins, after electrophoresis of aliquats of various protein fractions. The two arrows on the insert electropherogram indicate the relative mobility of the antigen. Protein fractions between the arrows on the elution profile (fraction numbers 18–28) were used for subsequent purification of the antigen.

Figure iii relates to the elution profile of *R. appendiculatus* proteins on DEAE-Sephacel column. Pooled protein fractions from sepharose CL–4B column were dialysed against 0.02M Tris–HCl, pH 8, concentrated by ultrafilteration and applied on DEAE–Sephacel column. The bound proteins were eluted with 30mM–250mM NaCl gradient in 0.02M Tris–HCl, pH 8.0, buffer. The absorbance of each protein fraction was monitored at 280nm (o—o) and 400 nm (o—o). The inset shows an electropherogram (regular PAGE) of various protein fractions eluted with a salt concentration between 130–150mM. the electrophoresed proteins were stained for proteins with Coomassie Brilliant Blue R.

Figure iv relates to Ouchterlony immunodiffusion showing identify of the purified antigen when reacted with antiserum raised in rabbits against whole female tick homogenate. Aliquats of various fractions eluted from DEAE–Sephacel column were tested by Ouchterlony immunodiffusion for immuno-reactivity with. Centre well: antiserum. Peripheral wells: a, protein fraction eluted with 66nM NaCl; b, fractions eluted with salt concentration between 130–150 mM; c, pooled and concentrated protein fraction after gel permeation; d, protein fractions eluted with 250nM NaCl from the DEAE-Sephacel column.

Figure v relates to regular PAGE of haemolymph from fully engorged adult male and female ticks, crude egg extract and the purified antigen.The electrophoresed protein bands were visualised by staining the gel with Coomassie Brilliant Blue–R. The various lanes contain proteins from: a, male haemolymph; b, female haemolymph; c, haemolymph from females irradiated with 2.4 Krad gamma-irradiation prior to attachment and feeding on the host; d, crude egg extract and e, the purified antigen. The extreme left and right lanes were loaded with Pharmacia high molecular weight protein standards. The molecular weights are indicated along the right margin.

Figure vi relates to SDS–PAGE of the antigen after DEAE-Sephacel ion-exchange chromatography. The gel was stained for proteins.Lanes a and b represent Pharmacia high and low molecular weight markers c, the purified antigen. The molecular weights of the Pharmacia standards is indicated along the left margin. P1–P8 represent sub-units of the antigen.

(i) *Preparation of antiserum and detection of major antigens*

(a) *Antiserum preparation*

Three hundred unfed adult females of *R. appendiculatus* were washed in distilled water and surface sterilized by immersion in 70% ethanol for 1 minute. The ticks were allowed to dry at room temperature and were then homogenized with a mortar and pestle in 5ml of PBS (100mM sodium phosphate, pH 7.2; 73mM NaCl) at 4°C. Purified sand (40–100 mesh, BDH Chemicals Ltd, Poole, England) was used to assist thorough disruption. The liquid obtained was centrifuged at 300xg for 30 minutes at 4°C. The supernatant was decanted and filtered through Whatman No. 1 filter paper. This filtrate was centrifuged at 10,000xg for 30 minutes at 4°C and the resulting supernatant was filtered through 0.45u filters (Falcon Sterile Disposable Filter units, Falcon, Oxnard Calif. USA). This extract was stored at − 20°C in2ml aliquots until used.

Fully fed adult female ticks were also homogenized, with an emulsifying stirrer, in PBS (50 ticks in 25ml PBS) at 4°C and then incubated at 4°C for 36 hours. The supernatant was centrifuged at 10,000xg for 30 minutes at 4°C. The resulting supernatant was collected and lyophilized in 2ml aliquots and stored at − 20°C. Protein concentration in both extracts was determined using standard methods.

Two rabbits (average weight of 3.5kg) were inoculated intramuscularly in the hind legs with 1.2mg protein equivalent/kg body weight unfed tick homogenate emulsified with an equal volume of Freund's Complete Adjuvant, FCA, (Difco Laboratories, Detroit, Mich., U.S.A.). The emulsion also contained antibiotics (50 units/ml penicillin and 50ug/ml streptomycin). The rabbits were challenged by having 100 larval ticks fed on them on the fourth week post inoculation. No adverse effects were noticed in the feeding performance of these infested larvae or on their subsequent development into nymphs.

After one year, these rabbits were reimmunized with the antigen preparation derived from fully fed female ticks. The rabbits were inoculated intramuscularly with 1.08 mg protein equivalent/kg. body weight of this homogenate emulsified with an equal volume of FCA. After 14 days the rabbits were against inoculated with the same amount of extract emulsified with FCA. The rabbits were boosted intramuscularly on day 28 with the same amount of extract in normal saline. On day 34 the animals were bled and sera prepared and stored at − 20°C. On

day 35 both the immunized and control animals (inoculated with saline in FCA and saline alone) were infested with 50 adult males and 50 adult females of *R. appendiculatus* to assess the effect of immunization on tick feeding and reproductive performance. The results of this treatment are given in Table 1 and may be compared with those obtained from animals which were repeatedly subjected to tick infestation (Table 2). In addition to the parameters listed in the tables, the ticks showed delayed attachment, prolonged tick feeding period, prolonged period for the replete ticks to drop-off the host and delayed time of oviposition.

The antisera from both groups of host animals were used to monitor the separation as purification of the said antigen.

TABLE 1

Engorgement weights, egg production and egg hatchability from R. appendiculatus infested on rabbits previously immunized with unfed tick homogenate and then reimmunized with homogenate from fully fed ticks one year later.

Rabbit[a]	Engorgement weight (mg±S.E.)	Total number of eggs produced per female tick (±S.E.)	% of eggs hatched into active larvae (± S.E.)
Control	374.7 ± 16.7	2614 ± 1 71	68.9 ± 1.8
Immunized	120.2 ± 26.8	1797 ±270	7.4 ± 1.8

a. Each group contained four animals

b. Ten egg batches from the 50 engorged female ticks fed on each rabbit were selected randomly for easier enumeration of hatching larvae

TABLE 2

Mean weight measurements of R. appendiculatus adults feeding on rabbits in four successive infestations. 50 females and 50 males were fed on each rabbit during each infestation

Number of infestation	Rabbits (Measurements in (mg ± S.E.)				Group Mean ± S.E.
	R_1	R_2	R_3	R_4	
1	344 ± 29	352 ± 37	314 ± 36	331 ± 27	335 ± 8
2	192 ± 9	195 ± 21	175 ± 13	183 ± 19	186 ± 5
3	113 ± 7	116 ± 10	119 ± 17	123 ± 22	179 ± 2
4	45 ± 3	47 ± 4	44 ± 4	46 ± 3	45 ± 1

Analysis of variance:

Source	df	SS	MS	F value
Groups	3	183368	61123	728***
Residuals	13	1095	84	
Total	16	184463		

(b) *Cross immunoelectrophoresis*

Crossed immunoelectrophoresis was performed using a standard method outlined as follows. Gels of 1.3mm thickness were prepared on glass slides from 1.2% (w/v) agarose in barbitone buffer (20.8mM sodium barbitone, pH 8.6, 2.3mM calcium lactate). 20ul samples (64mg protein per ml) of fully fed female tick antigen extract prepared as the radiolabelled antigen extract then dialysed into crossed immoelectrophoresis tank buffer also containing 0.1% NP-40 and centifuged for 1 hour at 100,000xg at 4°C, were electrophoresed for 1 hour at 2mA/cm. After electrophoresis the agarose strips (10 × 50mm) containing antigen were transferred to pre-coated 50 × 50mm glass plates. The remainder of the plate was filled to 1.3mm thickness with agarose containing antiserum (15ul per cm^2). Electrophoresis in the second direction was performed at 3mA/cm for four hours. Both electrophoreses were performed in a temperature regulated apparatus at 15°C. The gels were pressed, washed three times in PBS, once in distilled water, pressed again and then dried. The plates were stained in Coomassie Brilliant Blue.

The results of crossed immunoelectrophoresis are presented in Fig. i. A total of 12 tick antigens were detected (labelled 1-12 starting from the anode).

(ii) *Isolation and characterisation of the major antigens from eggs*

(a) *Isolation and purification*

Eggs, oviposited by fully engorged mated (FEM) adult female hard tick, *Rhipicephalus appendiculatus,* were collected every day, frozen in liquid N_2 and stored frozen at − 20°C. Soluble proteins were extracted by homogenizing eggs at 4°C in a TNE buffer (2:1 w/v; 0.15M Tris-HCl, 0.1M NaCl, 0.1M EDTA, 0.02% sodium azide, pH 8.0) containing 1mM PMSF and

10mM DTT. The homogenate was centrifuged at 10,000 rpm (Beckmann SW50.1 rotor) for 10 minutes. The supernatant was used in the subsequent steps for the purification of the antigen.

Gel Permeation Chromatography. This was performed on a 1.5 cm by 90 cm Sepharose CL–4B column equilibrated with TNE buffer without PMSF and DTT. Fractions (3.1 ml each) were collected and the absorbance of each protein fraction read at 280 and 400nm respectively by means of a Beckman SP500 spectrophotometer. Aliquots of various fractions, respresentative of the elution profile, were used for polyacrylamide gel electrophoresis (PAGE) [Fig. iii.

Anion Exchange Chromatography. Gel electrophoresis showed that approximately 34 ml of sepharose CL–4B eluate showed maximum absorbance at 400nm and also contained the antigen. The fractions were pooled and the volume reduced to 5.0 ml by ultrafilteration through a YM20 Amicon membrane. The concentrated protein fractions were dialysed against 20mM Tris–HCl, pH 8.0 and 3.0 ml applied to a 1.5cm × 30cm DEAE-sephaced column equilibrated in the same buffer. After washing to remove non-adsorbed protein, the bound proteins were eluted by means of a 30–250mM NaCl gradient (total volume 200ml) in the washing buffer. Fractions (3.1 ml each) were collected and the absorbance of the proteins in each fraction read at 280 and 400nm respectively. Gel electrophoresis of various fractions revealed that protein eluted with a salt concentration of 130–150 mM was predominantly the desired antigen [Fig. iii]. The antigen containing fractions, wre brownish in colour due to the covalent association of heme. These fractions were pooled, concentrated to 5.0 ml by ultrafilteration and dialysed against phosphate buffered saline (PBS, 0.03M sodium phosphate, 0.01M NaCl, 0.02% (w/v) NaN_3, pH 7.0)c)

(b) *Characterisation*

Ouchterlony Immunodiffusion Test. The antigen isolated from the above procedure reacted with the antiserum described earlier giving rise to a single precipitin line by Ouchterlony immunodiffusion test, indicating that it is one of the 12 antigens precipitated from the protein extracts of fully engorged mated females [Fig. iv).

Molecular Weight Estimation. The native molecular weight of the antigen was estimated to be 485,000 Dalton units by its electrophoretic mobility relative to those of standards on PAGE [Fig. v]. The sub-unit composition of the antigen and the molecular weights of the units were determined as above but in the presence of sodium dodecyl sulphate [Fig. vi]. Eight subunits were detected with molecular masses of 160, 137, 110, 100, 85, 79, 66 and 43 thousand Dalton units respectively.

PAGE. Electrophoresis (SDS–PAGE and regular PAGE) were performed on a 3–15% gradient gel slabs (1.5 × 18 × 20 cm) made by a BRL gradient maker. The gels were stained with Coomassie Brilliant Blue R–250 for protein, with periodate-Schiff reagent for glycoproteins or with Sudan Black B for lipoproteins. The native protein, as well as the subunits, gave positive reaction with all the three reagents showing that they were all glycolipoproteins.

Gel electrophoresis of the antigen preparation also reve led it to be the predominant band when the gel was stained for proteins. Haemolymph samples of both the sexes, and an aliquot of crude egg extract were also electrophoresed along with the purified antigen [Fig. v). A protein band, with similar electrophoretic mobility as the purified antigen was present in the egg extract and female haemolymph but not in the male haemolymph. These results were also confirmed by immunodiffusion. The isolated antigen is thus specific to female *R. appendiculatus.*

(iii) *Immunisation studies*

Each of a group of 5 mice were immunized by intradermal injection with a preparation containing about 500 ug of the said antigen with Freund's Complete Adjuvant in phosphate buffered saline (PBS) at pH 7.2. Booster immunizations were carried out after 2 and 4 weeks respectively.

Two weeks after the second booster, each mouse was challenged by 20 *R. appendiculatus* larvae placed in enclosed plastic capsule of 1.5cm diameter, attached with loctite superglue to a shaved region just below the neck of the mouse. The larvae were inspected daily for 7 days for any sign of attachment on engorgement.

The mice were each challenged again 5 months later with second sets of 20 larvae of *R. appendiculatus.*

In both assays, a count was made of attached and unattached larvae, of engorged larvae and of any dead, unfed larvae.

Table 3 gives a summary of the results obtained.

The results show that the effect of feeding on immunized mice soon after immunisation resulted in a high degree of larval mortality. On the other hand, 5 months later the effect of immunisation manifested itself in failure of the larvae to attach and feed

TABLE 3

Comparison of larval mortality and attachment on unimmunised mice and on mice immunized wtih a preparation conataining the antigen in Freuend's Complete Adjuvant

	1st Challenge (2 weeks after boosters)				2nd Challenge (5 months after boosters)			
	Larval Mortality				Larval Attachment			
	Control		Immunized		Control		Immunized	
Mice	Dead	Alive	Dead	Alive	Attached	Unattached	Attached	Unattached
1	3	15	10	10	20	0	1	19
2	0	7	17	3	20	0	5	15
3	1	6	18	2	20	0	2	18
4	4	16	9	11	20	0	17	3
Group Mean±S.E.	2.0±0.9.	11.0±2.6	13.5 ± 2.3	6.5 ± 2.3	20.0 ± 0.0	0.0	6.3 ± 3.7	13.8 ± 3.7

Referring to claims 5 and 6 below, suitable adjuvants are various combinations of substances such as alum, waxes, mineral oil, killed tubercle bacilli and any other readily metabolisable additive, synthetic or natural, known to enhance the persistence of antigens in the inoculated animal.

CLAIMS

1. A hemeglycolipoprotein of molecular weight of 484,000 Dalton units which is specific to female *Rhipicephalus appendiculatus* ticks and which is present as a major, soluble protein component of fully developed eggs of the ticks.

2. A glycolipoprotein derived from the hemeglycolipoprotein as described in claim 1 with the heme moiety removed.

3. The eight glycolipo- subunits derived from the native protein described in claim 1, with molecular weights of 160, 137, 110, 100, 85, 79, 66 and 43 thousand Dalton units, respectively, which may be used individually or in a selected combination.

4. A method of controlling the tick *Rhipicephalus appendiculatus*, the vector of the African cattle disease East Coast Fever which involves immunising cattle and other hosts with a preparation containing the native hemeglycolipoprotein described in claim 1, the glycolipoprotein as described in claim 2, or any combination of subunits that comprises this protein as described in claim 3.

5. An immunogenic preparation comprising as an active ingredient the native hemeglycolipoprotein described in claim 1, the glycolipoprotein as described in claim 2, or any combination of its subunits as described in claim 3, in association with a suitable adjuvant.

6. An immonogenic preparation comprising the hemeglycolipoprotein as described in claim 1, the glycolipoprotein as described in claim 2, or any combination of its subunits as described in claim 3, complexed with antibodies derived from animals immunised against *R. appendiculatus*, and the resultant complexes being formulated with suitable adjuvants as cited in claim 4.

7. Premixes of relatively high concentration of the antigens as defined in claims 1, 2 and 3 or of antigen-antibody complexes as defined in claim 5, formulated for ease of handling and transport, and which can be used as stock material for the preparation of the final immunogenic composition for use in the inoculation of the livestock and other host animals.

8. A hemeglycolipoprotein substantially as described herein in any of the examples.

9. A glycolipoprotein substantially as described in any of the examples.

10. A glycolipo- subunit substantially as described herein in any of the examples.

11. A method of controlling the tick *Rhipicephalus appendiculatus* substantially as described herein in any of the examples.

12. An immunogenic preparation substantially as described herein in any of the examples.

13. A premix of antigens substantially as described herein in any of the examples.

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